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TITLE: "Molecular Signatures of Chronic Pain Subtypes"

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13. SUPPLEMENTARY NOTES				
14. ABSTRACT This project continues to be a biomarker discovery program focusing on the causes of persistent pain after traumatic amputation in the combat setting. In the third year we have: 1) maintained the necessary regulatory approval at WRNMMC and Duke University. We have: 1) obtained and maintained documents for approval by MRMC; 2) completed patient enrollment at 124 patients; 3) maintained our interactive, secure web based data collection system; 4) populated our biorepository at Duke with bioresource collected from 124 patients enrolled at WRNMMC; 5) conducted further on-site visits and investigator meetings at WRNMMC; 6) Received all data from whole exome sequencing, gene expression, DNA methylation, miRNA, proteomic and metabolomic analysis of the 79 patient VIPER discovery cohort. We have also submitted one more paper for publication and presented two posters at pain medicine meetings. We have identified multiples pathways with biomarker and therapeutic analgesic relevance and received funding from the CDMRP Neurosensory FY 13 award to further study these pathways.				
15. SUBJECT TERMS Biomarker discovery, traumatic amputation, phantom limb pain, neuropathic residual limb pain, post-amputation limb pain				
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1. INTRODUCTION:

The primary purpose of this prospective cohort study project is to identify circulating biomarkers of persistent post-amputation pain among patients who develop persistent (greater than 3 months) phantom limb pain or neuropathic residual limb pain within three months (and less than 18 months) of their amputation as compared to those patients who do not. Patients with persistent post amputation limb pain have been assigned to the case cohort as determined by an expert panel within the research team utilizing the various pain scales (LANSS) or pain type questionnaires (PLP, RLP, CRPS).

The project aims include developing a systems biology derived model of neuropathic pain, such that the model is able to correctly assign class (i.e. case or control) in more than 95% of cases, and interrogation of the features which represent circulating qualitative and quantitative biomarkers of pain.

The primary outcome variable is the cumulative incidence of amputation pain in the first twelve months after injury related limb amputation. The project is divided into four tasks – human subject approval and enrollment, biomarker discovery, genotyping and re-sequencing for rare variant discovery

2. KEYWORDS:

Chronic Pain, neuropathic pain, novel analgesics, neuroinflammation, residual limb pain, phantom limb pain, pain subtypes.

3. OVERALL PROJECT SUMMARY:

Overall progress is reported according to the tasks laid out in the SOW. The SOW text is italicized, with the summary annual report text immediately following.

Study Task 1

We will enroll subjects between 3 and 18 months after amputation for traumatic injury in an observational study of different subtypes of post amputation chronic pain.

(a) Human subjects approval.

We expect this subtask to take 6-9 months. We will obtain IRB approval at Walter Reed Army Medical Center, in conjunction with our collaborator Dr Buckenmaier and his colleagues at DVPMI. We will submit the initial request for approval by the end of January 2011, and are advised the process is lengthy and may require several resubmissions. The first project milestone is thus IRB approval to enroll subjects at WRAMC, and we expect to reach this no later than 10/1/2011.

(b) Human subject enrollment.

We expect to enroll 165 amputee soldiers at WRAMC over the course of this 3-year project. The second project milestone is enrollment of the first subject by 12/1/2011. In order for the proteomic experiments to have sufficient power we need a minimum of 90 subjects. We will try and enroll as many subjects as we can in the first 12 months after IRB approval. There are several hundred

potentially eligible patients undergoing treatment at MATC as of December 2010. The third project milestone is thus enrollment of a minimum of 90 subjects by 12/1/2012.

Study Task 1 – Year 4 Summary Report

We completed enrollment of 124 patients. We have maintained a near 100% completion of the case report forms, with less than 1% missing data. In brief, there are 124 patients of whom 80 (64%) are cases and 44 (36%) controls. The incidence of residual limb pain is 72/124 (58%) and of phantom pain 72/124 (58%). The subtypes of residual limb pain are neuroma (37/124), mosaic (8/124), CRPS (15/124) and somatic (31/124). These incidences are entirely in keeping with the previously published rates.

Study Task 2 – Biomarker Discovery (Aims 1 & 2)

i. Proteomics

<i>Duration</i>	<i>12 months</i>
<i>Milestone</i>	<i>Final data back from Duke Core</i>

ii. Genotyping

<i>Duration</i>	<i>6 months</i>
<i>Milestone</i>	<i>Final data back from Duke Core</i>

iii. Sequencing

<i>Duration</i>	<i>9 months</i>
<i>Milestone</i>	<i>Final data back from Duke Core</i>

Study Task 2 – Year 3 Summary Report

Proteomic and Metabolomic Discovery Subtask (i)

We have paralleled our human biomarker and novel pathway discovery work in humans with a mouse peripheral nerve injury model that has been developed under the direction of another member of our lab, Dr. Thomas Van de Ven. This project utilizes funding from an NIH T32 training grant awarded to the Duke Department of Anesthesiology to produce a mouse peripheral nerve injury model that approximates the pathology present in human amputees. We have performed metabolomic and proteomic analysis of various mouse tissues from this model, including blood plasma, for cross-species verification of potential biomarkers of interest.

We have completed metabolome analysis on the VIPER discovery cohort and have used the resulting data, with cross-species verification in mice, to move forward on a possible new analgesic pathway known as TGR5. This project has been funded by a follow on DMRDP Neurosensory FY13 award.

Genotyping and Epigenetic Discovery Subtask (ii)

We have completed DNA methylation array analysis on the VIPER discovery cohort. This is an unprecedented dataset and represents an incredible opportunity to learn the mechanisms underlying the transition from acute to chronic pain in military amputees. We are currently working with our statistician, Dr. Yi-Ju Li, who is an Associate Professor in the Department of Biostatistics and Bioinformatics at the Center for Human Genetics in the Duke Department of Medicine. She and her postdoctoral candidate are performing the complex convergent pathway analysis required to complete our search for novel pain biomarkers and pathways. The initial results have led to identification of the wnt pathway as a putative analgesic pathway and the continuation of this work has been funded by the DMRDP award mentioned above. We are continuing targeted qPCR RNA and methylation analysis on our 45 patient validation cohort to confirm findings from the discovery cohort.

Whole Exome Sequencing Subtask (iii)

We have successfully performed whole exome sequencing on the entire 124 patient cohort and received all data back from the sequencing core facility. Initial analysis of this data has revealed several interesting pain pathways and biomarkers, including extremely significant clustering of Wnt pathway polymorphisms in amputees with pain compared to those without. The Wnt pathway has, very recently, been identified as a regulator of chronic neuropathic pain. In addition, we have completed overall pathway analysis on the exome sequencing data that is now ready for publication.

4. KEY RESEARCH ACCOMPLISHMENTS – YEAR 4:

- Completed patient enrollment at 124.
- Completed genome wide DNA methylation, gene expression, miRNA array, unbiased plasma proteomic and global plasma metabolomic data received on 79 patient discovery cohort and whole exome sequencing on all 124 patients
- Multiple putative pain pathways identified with two pathways chosen for directed mechanistic investigation. Each of these pathways (TGR5/FXR, Wnt and NOD1) may provide novel future therapeutic options.
- Omega-3 fatty acid levels from VIPER metabolomics correlated with post-amputation pain leading to testing of omega-3 fatty acid supplementation in mice with nerve injury. A publication describing this data has been submitted.
- Cytokine array analysis revealed significant differences in multiple cytokines between amputees with and without pain. These results will be submitted for publication in one month. These results were reported in a poster presented at the Pain Society of the Carolinas (PSOC) meeting in 2014.
- Validation analysis continues on 45 patient validation cohort.
- One abstract/poster presented at PSOC annual meeting. One abstract presented at AAPM annual meeting.
- Follow-on intervention study currently enrolling (PT110575, T Buchheit PI).
- Follow-on DMRDP Neurosensory Research Award (MR130082) funded to study the analgesic potential of the pathways discovered from VIPER data analysis.
- One additional paper submitted.

5. CONCLUSION:

We have completed project enrollment and collected an unmatched dataset already revealing several important pathways and biomarkers of interest. We have identified some surprising biology in the form of TGR5 and FXR regulation, NOD1 sequence variants and Wnt pathway change. We have received a DMRDP FY13 Neurosensory Research Award to study these novel pathways to advance our goal of developing novel analgesics to prevent and treat chronic residual limb pain.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

1. Peer-Reviewed Scientific Journals:

Submitted: Jacqueline Zillioux BS, Alexander Chamessian BS, Matthew Mauck MD, PHD, Thomas Buchheit MD, J. Will Thompson PhD, Chester Buckenmaier III MD, Andrew Shaw MB, FRCA, FCCM, Thomas Van de Ven MD, PhD. Perioperative supplementation with omega-3 fatty acids may attenuate post-surgical neuropathic pain. Pain Medicine (submitted)

2. Abstracts:

Abstract submitted and poster presented at PSOC conference 2014

- o Inflammatory Biomarkers in Patients with Persistent Post-operative Pain after Amputation

Abstract submitted and poster presented at AAPM Conference 2014

- o Regional Anesthesia Catheters Reduce the Severity of Neuropathic Post-Amputation Pain: Initial Results from the VIPER-80 Discovery Cohort of Injured Military Personnel

7. INVENTIONS, PATENTS AND LICENSES:

Not applicable

8. REPORTABLE OUTCOMES

Abstract presented at PSOC conference 2014

- o Inflammatory Biomarkers in Patients with Persistent Post-operative Pain after Amputation

Posters presented at AAPM Conference 2014

- o Regional Anesthesia Catheters Reduce the Severity of Neuropathic Post-Amputation Pain: Initial Results from the VIPER-80 Discovery Cohort of Injured Military Personnel

9. OTHER ACHIEVEMENTS:

Research Opportunity Applied for and Received

- o Follow-on DMRDP Neurosensory Research Award (MR130082) to study the analgesic potential of the pathways discovered from VIPER data analysis.

10. REFERENCES:

None

11. APPENDICES:

- Quad Chart
- Abstract (2)
- Publications (1)

Molecular Signatures of Chronic Pain Subtypes

Log# DM102142

Award Number W81XWH-11-2-0003



PI: Andrew D Shaw MD

Org: Duke University

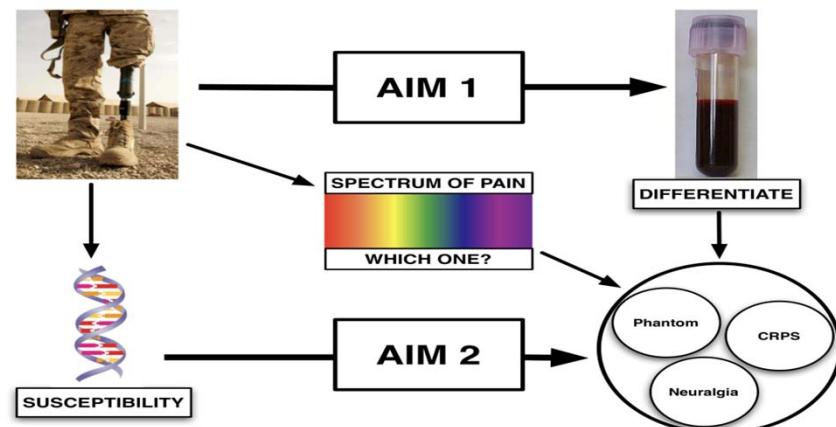
Award Amount: \$1,336,109.00

Study/Product Aim(s)

- Problem:** (1) There are no good tests to differentiate between different types of chronic amputation pain. (2) There are no good ways to measure susceptibility to chronic pain subtypes (phantom limb pain, neuralgia, CRPS).
- Hypothesis:** (1) Convergent pathway analysis from multiple data types including exome sequence, DNA methylation, gene expression, proteomics and metabolomics will reveal biomarkers of susceptibility to pain type.

Approach

- 1) Describe the molecular signatures of phantom pain, neuralgia pain and Complex Regional Pain Syndrome (CRPS) in 124 war injured amputees.
- 2) Describe the biology of chronic amputation pain subtypes in terms of the proteins and metabolites expressed in blood samples from amputees.
- 3) Generate a list of candidate risk genes for each subtype of amputation pain.
- 4) Sequence genes to find new polymorphisms associated with functional differences and risk of each pain subtype.



Accomplishment: We found significant increases in pro-inflammatory biomarkers (cytokines and chemokines) and significant decreases in anti-inflammatory biomarkers in patients with pain after amputation when compared to patients without pain.

Timeline and Cost

Activities	CY	11	12	13	14
Patient enrollment					
Circulating biomarker discovery					
Gene sequencing					
Validation Studies					
Estimated Budget (\$K)	\$481	\$449	\$406	NCE	

Goals/Milestones

CY14 Goal – Complete

- Complete patient enrollment
- All unbiased 'omic datasets collected.
- Received funding to further study pathways and biomarkers of interest found from this dataset. Neurosensory Research Award, MR130082 (PI: Van de Ven)
- Publication of manuscript describing the correlation between pain and plasma omega-3 fatty acid levels.

CY15 Goals

- Publication of clinical data, exome sequencing results, cytokine array data and methylation/expression results

Budget Expenditure to Date (as of 12 December 2014)

Projected Expenditure: \$1,336,109.00

Actual Expenditure: \$1,336,100.48

Updated: 01/09/2015



Inflammatory Biomarkers in Patients with Persistent Post-operative Pain after Amputation

Matthew Mauck, MD PhD¹; Alexander Chamessian¹; John Hsia, MD¹; Jacqueline Zillioux¹, David MacLeod, MB BS¹; Thomas Buchheit, MD¹; Chester "Trip" Buckenmaier III, MD²; Andrew Shaw, MB BS³; Thomas Van de Ven, MD PhD¹



¹Department of Anesthesiology, Duke University Medical Center and Durham VA Medical Center, Durham, NC 27710, USA

²Department of Anesthesiology, Walter Reed Medical Center, Uniform Services University

³Division of Cardiothoracic Anesthesiology, Department of Anesthesiology, Vanderbilt University Medical Center

Abstract:

- Persistent post-operative pain is one of the most feared outcomes in perioperative medicine.
- Patients undergoing amputation have a greater than 40% incidence of persistent pain after surgery
- 10% of those have pain that significantly alters functional status.
- The question of how acute post-surgical pain becomes chronic after amputation remains unresolved.
- In a post-amputation population, we explored the changes in plasma cytokine concentration that reflect systemic inflammatory state.

Methods:

- We used plasma samples from a prospective, cohort study that enrolled patients 3-18 months after amputation.
- Patients underwent objective pain testing, examination and blood sampling at the time of enrollment.

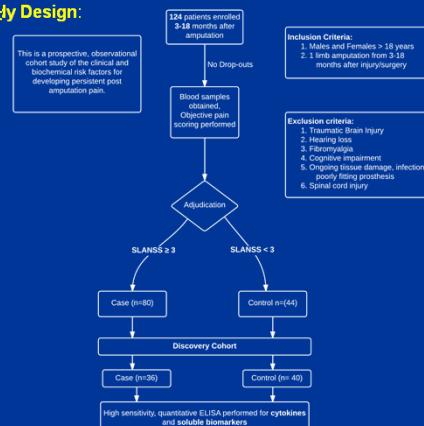
	Demographics Table		p-value
	Control (N=40)	Case (N=36)	
Age	25.20	27.69	0.1526*
Body Mass Index	25.66	26.78	0.1427*
Time since amputation	7.55	8.94	0.2243*
Smoking (ppd)	0.63	0.60	0.8168*
	N (%)	N(%)	
Male	40 (100)	35(97)	0.9577^
Smokers	25 (63)	21 (58)	0.8918^
Ethnicity	N (%)	N(%)	
American Indian/Alaska Native	0 (0)	0 (0)	1.0000^
Asian	2 (5)	1 (3)	1.0000^
Native Hawaiian or Other Pacific Islander	0 (0)	0 (0)	1.0000^
Black or African American	3 (7)	3 (8)	1.0000^
White	37 (93)	32 (89)	0.8836^

*p-value generated from a two-tailed t-test test. ^p-value generated from chi-squared test

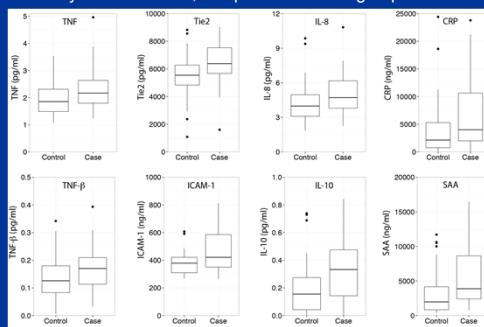
Patients with a SLANSS pain severity score of ≥ 2 were categorized as cases and patients with a pain severity score of < 2 were considered controls by a physician adjudication panel.

Plasma samples from the VIPER (Veterans Integrated Pain Experience Research) study were analyzed with a high-sensitivity enzyme-linked immunoassay (ELISA) for 37 soluble biomarkers including chemokines and cytokines that are involved in neuroinflammation.

Study Design:



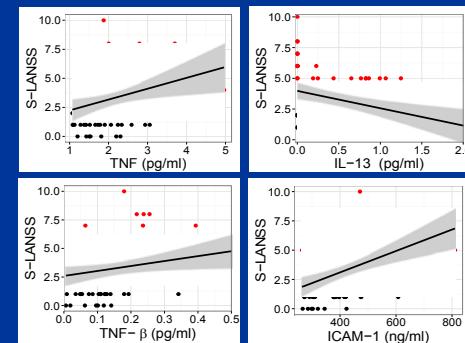
Results: Case subjects exhibited higher serum levels of the pro-inflammatory mediators TNF, IL-8, TNF- β , CRP, SAA, and Tie-2, and the anti-inflammatory mediator IL-10, compared to control group values.



Although these differences were statistically significant individually ($p < 0.05$), they did not withstand multiple comparison correction.

Results:

- There were several correlations between SLANSS severity score and Visual Analog Scale (VAS) score that remained significant even after adjustment for multiple comparisons.
- TNF- β and ICAM-1 were positively correlated with SLANSS score, while IL-13 exhibited a negative correlation.
- TNF and TNF- β were both positively correlated with VAS score.



Conclusion:

- Systemic inflammation after amputation is potentially a driver for the transition of acute pain to chronic pain. We observed an overall increase in inflammatory cytokines in cases versus control, suggesting that systemic inflammation has a role in the development and maintenance of persistent pain after amputation.
- We observe a negative correlation between SLANSS severity score, an indicator of neuropathic pain, and IL-13 indicating that anti-inflammatory cytokines may be protective in the development of chronic pain after amputation.
- This data has led us to hypothesize that pro-inflammatory cytokines drive persistent pain while anti-inflammatory cytokines, such as IL-13, serve a protective role. We additionally found elevations of ICAM-1 in cases relative to controls and a positive correlation with S-LANSS severity score, suggesting that leukocyte trafficking may enhance neuropathic pain phenotype.
- These results, while interesting, need follow-up in a larger scale, prospective study and validation in animal models of persistent pain before these biomarkers are seen as heralding chronic pain after surgery.



Regional Anesthesia Catheters Reduce the Incidence of Neuropathic Post-Amputation Pain: Results from the VIPER Cohort of Injured Military Personnel

Hung-Lun J. Hsia MD, Thomas Buchheit MD, Thomas Van de Ven MD PhD, David MacLeod, MB FRCA, Mary McDuffie RN, William White MS, COL Chester "Trip" Buckenmaier MD, and Andrew Shaw MB FRCA

Departments of Anesthesiology, Duke University Medical Center, Walter Reed National Military Medical Center, and Durham Veterans Affairs Medical Center



Background

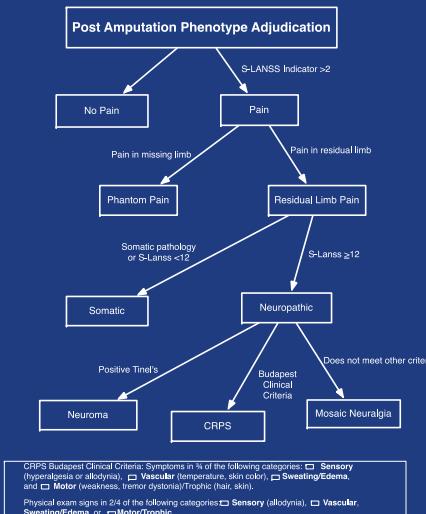
- Chronic pain is a common problem in injured military service members undergoing amputation.¹
- Most studies of post-amputation pain only discriminate phantom and residual limb pain.²
- Sub-classification of pain phenotypes is an important step in the development of disease-specific therapies.
- An ongoing collaborative study (Veterans Integrated Pain Evaluation Research (VIPER)) between Duke University, Walter Reed National Military Medical Center (WRNMMC) and the Durham VAMC is being conducted to further define post-amputation clinical phenotypes and discover circulating biomarkers of persistent pain.
- Here we make a report on 124 military service members who have undergone clinical assessment and phenotypic adjudication.

Methods

Phenotypic Assignment

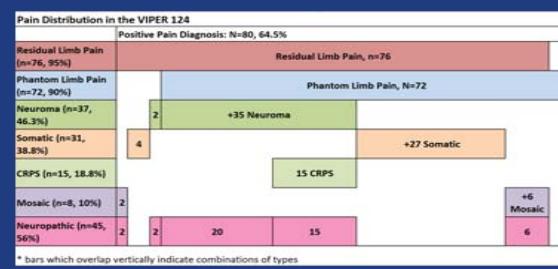
After IRB approval, the VIPER clinical cohort was assessed using validated questionnaire instruments:

- Brief Pain Inventory (BPI)
- Self-Reported Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale (S-LANSS)
- Complex Regional Pain Syndrome (Budapest Clinical Criteria)
- Phantom and residual limb pain questionnaires
- A formal endpoint adjudication was performed using the algorithm previously reported by our group³
- Phantom and residual limb pain were discriminated.
- Residual limb pain was then sub-categorized into a) Neuroma b) CRPS c) Mosaic Neuralgia or d) Somatic.

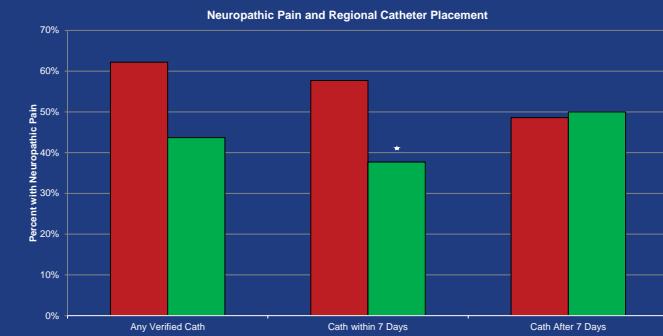


Results

- Using the Duke Post-Amputation Pain Algorithm (Duke PAPA), we discriminated between several post-amputation pain subtypes in military service members.
 - We found an overall incidence of post-amputation pain (PAP) of 64.5%.
 - When these PAP cases were further sub-categorized:
 - 90% described phantom pain
 - 95% described residual limb pain (RLP)
 - There was significant overlap with these diagnoses, but they did not always co-exist
- Furthermore, of those subjects with RLP the following diagnostic categories were noted:
 - 46.3% neuroma
 - 18.8% CRPS
 - 10% Mosaic neuralgia (neuralgic pain not otherwise specified)
 - 38.8% somatic
- In our analysis of retrospective catheter placement data, we found a significantly decreased incidence of neuropathic pain in patients receiving regional catheters within 7 days of injury.



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Conclusions

- We observed phenotypic complexity of post-amputation pain symptoms in this cohort including:
 - Strong overlap in the diagnoses of phantom and residual limb pain
 - Several distinct subtypes of residual limb neuropathic pain
 - A predominant contribution of neuroma symptoms in service members with residual limb pain
- Additionally, we observed that the use of early regional anesthesia catheters was associated with a decreased incidence of chronic neuropathic pain.

References

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- Ephrath PL, Wegener ST, Mackenzie EJ, Dillingham TR, Pezzin LE. Phantom pain, residual limb pain, and back pain in amputees: results of a national survey. *Arch Phys Med Rehabil*. Oct 2005;86(10):1910-1919.
- Lindsay DR, Pyati S, Buchheit TE, Shaw A. Residual limb pain: more than a single entity? *Anesthesiology*. Jan 2012;116(1):224.

Supported by Department of Defense (DM102142)

Title: Perioperative supplementation with omega-3 fatty acids may attenuate post-surgical neuropathic pain.

Jacqueline Zillioux BS, Alexander Chamessian BS, Matthew Mauck MD, PhD, Thomas Buchheit MD, J. Will Thompson PhD, Chester Buckenmaier III MD, Andrew Shaw MB, FRCA, FCCM, Thomas Van de Ven MD, PhD.

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Acknowledgements: none

Disclosures/Conflicts of Interest: none

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ABSTRACT

Objective: The omega-3 fatty acids docosahexaenoic (DHA) and eicosapentaenoic (EPA) are precursors to a family of analgesic and neuroprotective small pro-resolution lipid mediators (PRLMs) that include the resolvins and neuroprotectins. We hypothesized that perioperative supplementation with DHA and EPA can prevent post-surgical pain by increasing endogenous levels of PRLMs.

Methods: To identify targets for novel analgesics, our lab conducted a global metabolomics study of 80 human patients with traumatic amputations as part of the Veterans Integrative Pain Evaluation Research clinical trial. We analyzed the results of this study for associations between omega-3 fatty acids and pain severity. We then treated nerve-injured mice with perioperative oral DHA and EPA with or without aspirin to determine whether DHA, EPA and their PRLM metabolites reduce mechanical allodynia in a mouse model of peripheral nerve injury.

Results: There was a negative correlation between DHA and EPA concentration and neuropathic pain severity in human traumatic amputees. We found that mice treated with both DHA/EPA or DHA/EPA with aspirin had significantly reduced mechanical allodynia in the ipsilateral paw compared to injured control animals. There was no significant difference in allodynia reduction between the treatment groups. Also, there was a trend toward increased plasma PRLMs neuroprotectin D1 and protection DX in mice treated with DHA and EPA with aspirin.

Conclusion: Our results suggest that perioperative DHA/EPA supplementation may provide a safe, inexpensive and effective way to increase PRLM levels and prevent chronic pain in humans.

Keywords: Chronic postoperative pain, inflammation, neuropathic pain, omega-3 fatty acids

INTRODUCTION

Surgical procedures, including mastectomy, amputation, and thoracotomy, are followed by severe and disabling chronic neuropathic pain in 5-10% of cases (1). This post-surgical neuropathic pain and other chronic pain syndromes represent an enormous public health burden, annually costing the United States upwards of \$630 billion in direct health expenses and lost productivity (2). Despite our success at managing acute post-surgical pain, current therapeutic options for the chronic pain that follows are limited. As such, there is an immediate need for novel preventive analgesics.

Chronic neuropathic pain is believed to partially result from neuroinflammation following nerve injury and subsequent peripheral and central sensitization that occurs as a consequence of this inflammation (1-3). As it has become increasingly appreciated that initiation and resolution of inflammation are distinct processes (4), novel therapeutic strategies aimed at interrupting the inflammation and switching to a resolving state seem especially promising.

Growing evidence suggests that the novel pro-resolving lipid mediator (PRLM) metabolites of the omega-3 fatty acids docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) may achieve this aim. These oxylipins, which include resolvins, neuroprotectins, and maresins, are generated via several metabolic pathways involving lipoxygenase (LOX) or cyclooxygenase (COX) enzymes at sites of tissue injury (5,6). Of note, there are two R-series resolvins that are generated from DHA and EPA by the

aspirin acetylated COX2 enzyme (5). Pro-resolving oxylipins have proven potent anti-inflammatory agents: for example, resolvins are 1000 times more effective than DHA or EPA and 100 times more effective than morphine at mitigating inflammatory pain (7). Resolvins and neuroprotectins have been extensively studied in multiple rodent models of pain and found to prevent as well as treat established inflammatory, post-surgical, and neuropathic pain (6,10). These effects are attributed to their ability to actively resolve inflammation as well as inhibit neural plasticity, glial activation, and transient receptor potential (TRP) channels (8-10).

The pro-resolving properties of resolvins and neuroprotectins likely explain the wide therapeutic applications of omega-3 polyunsaturated fatty acids (PUFAs). Therapeutic benefit of supplementation with omega-3 PUFAs has long been suspected in inflammatory and cognitive diseases (11,12), and has been well established in the case of heart disease despite incomplete understanding of its underlying mechanisms (13,14). By providing the raw material to enhance PRLM production at the site of injury, omega-3 fatty acid supplementation may act as a safe and novel preventive analgesic therapy.

We have recently completed enrollment of one hundred and twenty four recent active duty military post-traumatic amputees at Walter Reed National Military Medical Center receiving care at the Defense and Veterans Center for Integrative Pain Medicine (DVCIPM) under the Veterans Integrated Pain Evaluation Research Study (VIPER). Patients were enrolled three to eighteen months after amputation. Each patient was categorized as "case" or "control" based on S-LANSS severity score and blood samples were drawn for multiple types of data analysis, including global metabolomics profiling

that included quantification of plasma fatty acids allowing correlation with post-amputation pain scores.

In addition, we use a murine spared-nerve injury model to test whether perioperative supplementation with omega-3 PUFAs: (A) Increases endogenous levels of pro-resolving oxylipins in mice with co-existing peripheral nerve injury, (B) attenuates chronic neuropathic pain in a mouse model of peripheral nerve injury and (C) if the addition of aspirin augments pro-resolving oxylipin levels and improves pain relief.

METHODS

VIPER Study Design and sample collection

After IRB approval, 124 subjects were enrolled at Walter Reed National Military Medical Center (WRNMMC) in this retrospective cohort study. Multiple pain and psychometric questionnaires were administered to each individual to be completed with minimal guidance by a healthcare provider. One of the included questionnaires was the self-report version of the Leeds Assessment of Neuropathic Symptoms and Signs score (S-LANSS), which includes a question asking each patient to report average pain severity in the affected limb over the past week. We termed this question the S-LANSS severity score. Patients were designated as "cases" if they had an S-LANSS severity score greater or equal to 3.

Subjects were included if they were a military health care beneficiary age 18 years or older and undergoing treatment at WRNMMC with a diagnosis of post injury amputation of all or part of one limb. Amputation injury must also have occurred between 3 and 18 months prior to enrollment.

Patients were excluded if they were afflicted with severe traumatic brain injury, significant cognitive deficits, substantial hearing loss, spinal cord injury with permanent or persistent deficits, ongoing tissue damage pain, infection, bone spur, poorly fitting prosthesis, or hip disarticulation. Blood samples were collected in EDTA containing tubes, centrifuged for 15 minutes at 1600G at 4°C within ~30 minutes of sample collection, and plasma pipetted off and aliquoted into 1.8ml cryovials.

Unbiased Plasma Metabolomics

Metabolomics experiments were conducted by Metabolon Inc (Durham NC) Sample Preparation: The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions: one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. Essential fatty acid sub-analysis required the LC/MS platform.

Liquid chromatography/Mass Spectrometry (LC/MS, LC/MS²): The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots,

dried, then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% Formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM Ammonium Bicarbonate. The MS analysis alternated between MS and data-dependent MS² scans using dynamic exclusion.

Accurate Mass Determination and MS/MS fragmentation (LC/MS), (LC/MS/MS): The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which had a linear ion-trap (LIT) front end and a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer backend. For ions with counts greater than 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 ppm. Ions with less than two million counts require a greater amount of effort to characterize. Fragmentation spectra (MS/MS) were typically generated in data dependent manner, but if necessary, targeted MS/MS could be employed, such as in the case of lower level signals.

The human plasma dataset comprised a total of 363 named biochemicals and 295 unnamed compounds. Following log transformation and imputation with minimum observed values for each compound, a One Way ANOVA with Contrasts and a Welch's

t-Test were used to identify biochemicals that differed significantly between experimental groups

Animals

All animal experiments were approved by the Institutional Animal Care & Use Committee at Duke University and were conducted in accordance with the U.S. Government Principles for Utilization and Care of Vertebrate Animals for Testing, Research, and Training. 8-10 week-old male C57BL/6 mice ordered from Charles River Laboratories (Wilmington, MA) were used.

The mice were housed in cages of 5 and had access to chow and water ad libitum. The chow was either Picolab® Rodent Diet 20 (5053) or Rodent Laboratory Diet (5001) from LabDiet® (St. Louis, MO), both of which contain omega-3 to omega-6 ratios of about 0.15.

Drugs and Drug Administration

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) were purchased from Cayman Chemical as solutions in ethanol. For these experiments, after evaporating the ethanol solvent under a gentle nitrogen stream, DHA and EPA were delivered in a soybean oil control vehicle (Crisco Pure Vegetable Oil) containing the anti-oxidant Vitamin E. Soybean oil was chosen as the control vehicle for its low omega-3 to omega-6 PUFA ratio of 0.14 (15), which is similar to the mice's background diet. Acetylsalicylic acid (ASA) was purchased from Sigma.

All treatments were delivered daily to the mice via oral gavage, on the day before surgery through post-operative day 12. Mice in the appropriate treatment groups received approximately 200 mg/kg of DHA/EPA and 30 mg/kg of ASA per treatment. Dosing estimates are based on a 26g mouse, the average mass of an adult C57BL/6 mouse. Control groups received soybean oil over the same time span. The DHA and EPA were delivered together in the soybean oil carrier. ASA was delivered in water.

Spared-Tibial Nerve Injury Surgery

Spared-tibial nerve injury (SNI) surgery was performed under 2-3% isofluorane anesthesia, as described by Shields (16). The left hind limb was immobilized in a lateral position. After skin incision at the mid-thigh level and dissection through the underlying muscle, the sciatic nerve trifurcation was exposed. The common peroneal and sural nerve branches were tightly ligated with 6-0 silk sutures and then severed. Throughout the procedure, the tibial nerve was preserved by carefully avoiding any stretch or nerve contact. For sham surgeries, the sciatic nerve trifurcation was exposed without imposing any nerve injury.

Whole blood was collected from anesthetized mice (3-4% isofluorane) via cardiac puncture. To puncture the heart, a 23-gauge needle was inserted left of and under the sternum in the direction of the head at a 25 degree angle. Once blood was seen in the syringe, negative pressure was applied to the syringe. The whole blood was collected in a 1-mL EDTA tube and centrifuged at 5000 rpm for 5 minutes at room temperature. Plasma was then collected from the top of the centrifuged tube and stored at -80C.

Behavioral Assays

Mice were habituated to the testing environment for 2 days prior to baseline testing and for at least 30 minutes on each subsequent test day. The mice were placed in plastic boxes on an elevated wire-mesh apparatus. Mechanical allodynia was assessed by stimulating the left hind paw with Von Frey filaments of logarithmically increasing stiffness (0.04 – 2.00 g, Stoelting Co, Wood Dale, IL), applied perpendicularly to the plantar surface. Specifically, the hindpaw was stimulated in the distribution of the tibial nerve, in the center of the plantar surface (17). The 50% paw withdrawal thresholds were determined using the up-down method of Dixon (18). Testing was performed by a blinded researcher at baseline, post-operative day (POD) 3 and every following third day, finishing on POD 21.

Plasma Oxylipin Assays

Plasma oxylipin assays were performed at the Duke Proteomics and Metabolomics Shared Resource. Stable Isotope Labeled (SIL) oxylipin standard solutions were purchased from Cayman Chemical (Ann Arbor, MI). Solutions were combined in a stock SIL mixture in methanol which was further diluted with acetonitrile to a final concentration of 6.25 nM (IS Working Solution). Analytical standard solutions were purchased from Cayman Chemical (Ann Arbor, MI). Solutions were combined in a stock mixture containing 1 µg of each compound in methanol which was further diluted with 1:1 acetonitrile:methanol to prepare Spiking Solutions from which Quality Control samples (QC) and calibration standards were made. Calibration standards and QCs were prepared in 50 mg/mL Bovine Serum Albumin (BSA) in 50 mM ammonium

bicarbonate (AmBic). Calibration standards were analyzed in duplicate bracketing the study samples and QCs. The concentrations of the calibration standards were 10, 25, 50, 100, 250, 500, 1000, 5000, 10000, and 50000 pg/mL. QC samples were prepared at three concentrations 40000, 4000, and 400 pg/mL. These were analyzed in duplicate with the study samples.

Samples were extracted by protein precipitation with acetonitrile using a Biotage (Uppsala, Sweden) PLD+ protein and phospholipid removal 96-well plate. Plasma samples were thawed, mixed, and spun at a slow speed to pellet any solids. For each blank, calibration standard, QC, and study sample 800 µL IS Working Solution were added to the appropriate well of the protein and phospholipid removal 96-well plate. 800 µL acetonitrile were added to each well to be used for double blanks. Aliquots of 90 µL blank, calibration standard, and QC sample were added to the appropriate wells. Plasma study samples were added in 90 µL aliquots when possible. The extraction plate was then capped, mixed for 10 minutes at room temperature, and frozen for 10 minutes at -20°C. The collection 96-well plate, containing a solution with 5 ul glycerol as carrier, was positioned below the extraction plate in a vacuum block, then vacuum was applied for 5 minutes to elute the samples. The collected samples were dried under a gentle stream of nitrogen then reconstituted in 50 µL 1:1 acetonitrile:methanol. 5 µL were injected for LC/MS/MS analysis.

LC-MS/MS analysis of oxylipin molecules was performed based on the method of Laiakis et al [site <http://www.ncbi.nlm.nih.gov/pubmed/25126707>]. Briefly, UPLC separation was performed using a Waters (Milford, MA) Acquity UPLC using an Acquity 2.1 mm x 10 mm 1.7 µm BEH C18 column. Mobile phase A was water with 0.1% acetic

acid and mobile phase B was 90:10 acetonitrile:isopropyl alcohol. Samples were introduced directly into a Xevo TQ-S mass spectrometer (Waters) using negative electrospray ionization operating in the Multiple Reaction Monitoring (MRM) mode. MRM transitions (compound-specific precursor to product ion transitions) for each analyte and internal standard were collected over the appropriate retention time. The MRM data were imported into Waters application TargetLynx™ for peak integration, calibration, and concentration calculations. Analytes for which analytical standards were not included were quantified against the standard curve of an analyte from the same or similar compound class.

Statistical Analysis

Essential fatty acids concentrations from the metabolomics dataset were chosen for further analysis using GraphPad Prism 6 software (GraphPad Software, San Diego). Linear regression analyses were performed to assess the relationship between S-LANSS severity score and the plasma levels of omega-3 PUFA and omega-6 PUFAs. Patients with S-LANSS severity scores of 9 and 10 were excluded from this analysis, as there was only 1 patient per group.

Statistical analysis of mouse behavioral data was also performed in GraphPad. Paw withdrawal thresholds were normalized to baseline and are presented as mean percent of baseline with standard error of the mean. Missing data was imputed with the mean of the shared treatment group's paw withdrawal threshold for the time point. The paw withdrawal thresholds of the treatment groups were compared using repeated-measures two-way ANOVA and ad-hoc Tukey tests corrected for multiple comparisons.

Area under the curve and quadratic polynomial regression analyses of the paw withdrawal thresholds were also performed, the details of which are available in the Supplementary Methods.

Oxylipin assay results were also analyzed in GraphPad. One outlier was excluded from each of the SNI control and DHA/EPA treatment groups. Values below the lower limit of quantification (LLQ) were imputed with the LLQ, thereby biasing towards the null hypothesis. We pre-specified comparisons of the DHA/EPA groups with and without aspirin with the SNI control group. These comparisons were made using unpaired t-tests.

RESULTS

Plasma omega-3 PUFA concentration and omega-3 to omega-6 PUFA ratio are negatively correlated with severity of chronic post-amputation pain in humans

To assess the relationship between omega-3 PUFA plasma levels and pain in the VIPER patients, we performed linear regressions of DHA, EPA, and their summed plasma levels versus S-LANSS severity score (Figure 1A,B). The analyses showed significant negative correlation between both individual or summed omega-3 levels and S-LANSS severity score. The same analysis was done using the omega-6 PUFAs arachidonic acid, linoleic acid, and n-6 docosapentaenoic acid (DPA n-6) (Figure 1C). No significant correlations were found between S-LANNS severity score and omega-6 PUFA levels. We were also interested in the relationship between the ratio of omega-3 to omega-6 PUFAs and pain in the VIPER patients experiencing pain (Figure 1D).

Linear regression revealed a significant negative correlation between omega-3 to omega-6 PUFA ratio and S-LANSS severity score.

Perioperative supplementation with DHA and EPA attenuates post-nerve injury mechanical allodynia in mice.

To test the effect of DHA and EPA supplementation with and without aspirin on mechanical allodynia in mice with surgically induced peripheral nerve injury, we created 4 groups of 10 mice each. One group underwent sham surgery and received daily control soybean oil. The remaining groups all underwent SNI surgery and received daily control soybean oil, DHA and EPA, or DHA, EPA, and aspirin.

We assessed the influence of omega-3 PUFA supplementation on post-operative allodynia by comparing the groups' mean paw withdrawal thresholds across all time points (Figure 2). As anticipated, the SNI control mice developed significant mechanical allodynia by POD3 ($p<0.0001$) that peaked on PODs 9-12 and resolved by POD21. We found that treatment with DHA and EPA significantly attenuated the development of mechanical allodynia compared to SNI controls. The DHA/EPA group had improved allodynia compared to SNI on PODs 6, 9, and 15 (>50%, $p<0.05$), and trended towards improved outcome on POD12 (35.2%, $p=0.055$). Similarly, the treatment group with aspirin had substantially improved allodynia compared to SNI on PODs 3 through 12 (>50%, $p<0.05$). Aspirin did not appreciably augment the therapeutic effect of omega-3 PUFA supplementation, as there were no significant differences between the two treatment groups. We also performed area under the curve and quadratic regression analyses of the paw withdrawal thresholds to confirm the effect of omega-3

supplementation on mechanical allodynia. These analyses corroborated the above results, indicating that omega-3 supplementation interfered with the development of robust allodynia seen in SNI controls. (See Supplementary Materials for details).

Perioperative supplementation with DHA and ASA may augment plasma protectin DX (PDX) and neuroprotectin D1 (NPD1) in mice. (Figure 3)

We next created groups of mice identical to those described above to test the effect of DHA, EPA, and aspirin supplementation on endogenous levels of pro-resolving oxylipins. Plasma samples collected from 5 mice per group on POD12 were analyzed using an LC-MS/MS assay.

There was a trend towards increased levels of the DHA-derived oxylipins protectin DX (PDX) and neuroprotectin D1 (NPD1) in the treatment group treated with aspirin compared to the SNI controls (116.4 ± 54.7 pg/mL and 90.3 ± 49.3 pg/mL; $p=0.07$ and $p=0.11$, respectively). There was no significant difference between the DHA/EPA treatment group and the SNI controls. Resolvin D1, resolvin D2, and maresin plasma levels were all below the assay's LLQ and could not be quantified.

DISCUSSION

Mechanistic understanding of the transition from acute to chronic neuropathic pain after nerve injury has advanced significantly over the past decade, but advances in preventive therapeutics continue to be slow. In response, there has been increasing focus since 2010 on public/private partnerships to advance preventive analgesic discovery by standardizing pre-clinical and clinical analgesic studies (19). Recent

evidence that DHA- and EPA-derived small lipid mediators such as neuroprotectin D1 can provide preventive analgesia in animal models of post-nerve injury pain has provided one of the most intriguing leads toward novel preventive therapeutics (6,20). The PRLMs themselves are short lived and difficult to produce in quantity, but supplementation with their omega-3 fatty acid precursors offers a possible way to increase the concentration of these small lipid mediators at the target injury site. In addition, aspirin acetylation of cyclooxygenase has been shown to increase the production of a group of aspirin derived PRLMs that may further increase the analgesic efficacy of DHA/EPA supplementation (5, 21).

In this study we found that the plasma concentrations of DHA and EPA in young, recent active duty military amputees negatively correlated with chronic post-amputation pain severity. It is unclear from this data whether reduced plasma DHA and EPA is a result of having already existing chronic pain or whether those patients with diets high in omega-3 fatty acids are less likely to develop severe chronic pain after surgery, but this intriguing correlation prompted us to study whether supplementation of DHA/EPA in mice undergoing peripheral nerve injury (including the nerve transection that occurs in amputation) would similarly reduce post nerve injury allodynia and whether this supplementation increased plasma PRLM levels. Therefore, we provided oral supplementation of DHA and EPA to mice before and after spared-tibial nerve injury and found that these injured mice had significantly reduced post-injury mechanical allodynia and a trend toward higher plasma neuroprotectin levels. This reduction in allodynia continued for 6 days after oral DHA/EPA supplementation was discontinued. By day 21 all four experimental groups returned to baseline. Though allodynia reduction

in the two DHA/EPA treatment groups was dramatic, it is difficult to conclude from this data whether supplementation acts as a therapeutic or as a preventive therapeutic due to the inherent improvement of mechanical allodynia over time in this particular peripheral nerve injury model. There are several models of peripheral nerve injury that produce more dramatic and long-lasting allodynia that could be used in future studies to verify that DHA/EPA supplementation is preventive and not just therapeutic (22).

To determine whether DHA/EPA supplementation led to measurable increases in plasma PRLMs, we collected blood plasma at post-operative day 12 from mice treated in an identical manner to the four groups tested for mechanical allodynia. After LC/MS separation and analysis, we found a trend towards higher plasma concentrations of two neuroprotectins, NPD1 and PDX. NPD1 has been well studied as an analgesic and neuroprotective agent and is one of the few compounds found to provide preventive analgesia in a mouse model of peripheral nerve injury (10,20,23). PDX has been less well studied in animal pain models, but has been shown to block neutrophil infiltration in a mouse model of peritonitis (24). Though plasma concentration changes of these two lipids did not reach significance (defined as p-value of 0.05), the trend toward neuroprotectin levels is intriguing. Future studies would include increased sample size and volume, along with analysis of injured peripheral tissues where these lipids are likely formed. Analysis of affected tissue with higher concentrations of these mediators may also allow evaluation of resolin and maresin concentration changes with DHA/EPA supplementation, as the amounts of these PRLMs were below the lower limit of quantification in this study. Our study also included an aspirin treatment group since there are multiple aspirin-triggered PRLMs produced by the aspirin acetylated COX2

enzyme (5). However, aspirin treatment did not appear to enhance the reduction of mechanical allodynia in nerve-injured mice, and the effect of aspirin on the endogenous production of 18R-E series resolvins was not measurable since all resolvins in this study were present below the lower limit of quantification.

Though this pilot study did not definitively show that DHA/EPA supplementation increases plasma PRLMs, previous studies have demonstrated that supplementation with DHA and EPA translates to increased endogenous resolin levels in healthy volunteers (21,25). Also, there is growing evidence that increasing the ratio of omega-3 to omega-6 fatty acids likely augments the beneficial effects of omega-3 PUFA supplementation on multiple disease states (26). Recent work by Ramsden et al supports the idea that dietary supplementation with omega-3 fatty acids increases the blood concentration of these small lipid mediators while reducing pain symptoms (27). This clinical trial concluded that migraine patients receiving a high omega-3 and low omega-6 fatty acid diet had significantly higher blood levels of the immediate precursors to resolin and neuroprotectin biosynthesis and also increased resolin D2 concentration. They also found that this dietary intervention reduced the incidence of migraine headache. Reducing omega-6 fatty acids in the mouse diet in addition to DHA/EPA supplementation may produce more dramatic improvements in mechanical allodynia and small lipid mediators. This will have to be evaluated in future preclinical studies.

Conclusions:

Our results suggest that perioperative DHA/EPA supplementation may provide a safe, inexpensive and effective way to increase pro-resolving lipid mediator levels and

prevent chronic pain in humans. Given the low-risk of side effects and current high prevalence of use, omega-3 PUFAs supplementation could easily be applied perioperatively. A larger preclinical trial with increased sample size and an added experimental group with low omega-6 fatty acid diet is needed to confirm these findings. A clinical trial in keeping with the FDA Critical Path initiative and ACTION committee goals would be an ideal next step after preclinical confirmation.

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Disclosures/Conflicts of Interest: none

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Figure 1. Essential fatty acids and neuropathic pain in traumatic amputees

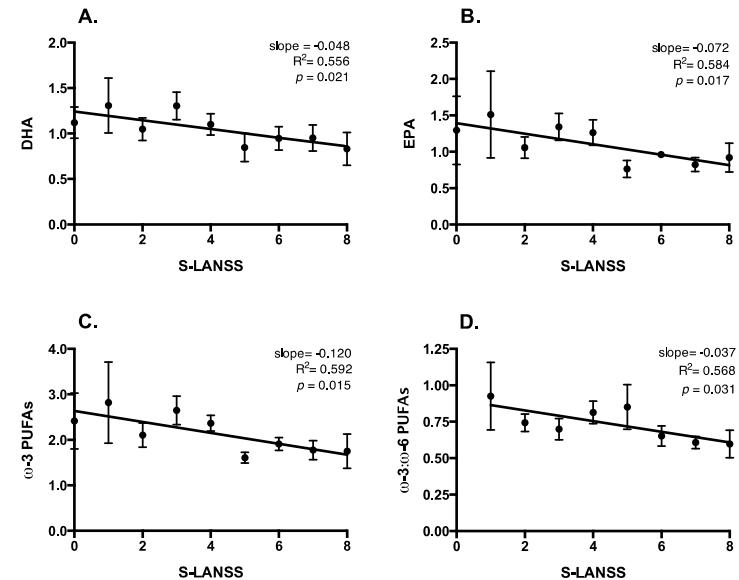


Figure 1: A-C: Plasma levels of the omega-3 fatty acids DHA and EPA are negatively correlated with neuropathic pain score in patients status-post traumatic amputation. Depicted are the summed or individual levels of the omega-3 polyunsaturated fatty acids (PUFAs) DHA and EPA in the plasma of 78 VIPER patients against the patients' S-LANSS severity score. Linear regression revealed significant negative correlation ($p<0.05$) between plasma omega-3 PUFA levels and pain score. D: There was also a negative correlation between the ratio of omega-3 to omega-6 PUFAs and neuropathic pain score. The essential fatty acids levels are relative: they represent raw area counts from the LC/MS analyses that have been normalized such that the cohort has a median of 1.

Figure 2. Oral DHA/EPA supplementation reduces mechanical allodynia

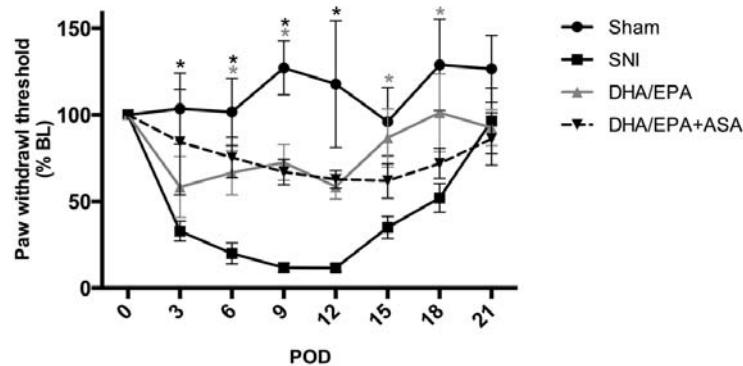


Figure 2: Perioperative supplementation with DHA and EPA attenuates mechanical allodynia following spared-nerve injury. Paw withdrawal thresholds are presented as mean percent of baseline \pm SEM, from baseline (POD0) through post-operative day (POD) 21. Lower thresholds represent mechanical allodynia. Mice treated with DHA and EPA (DHA/EPA, DHA/EPA + ASA) developed significantly less mechanical allodynia (50-60%) at the majority of time points compared to the spared-nerve injury control group (SNI). * represents significant difference of treatment group from SNI ($p<0.05$).

Figure 3. Perioperative supplementation with DHA/EPA may increase plasma PLRMs

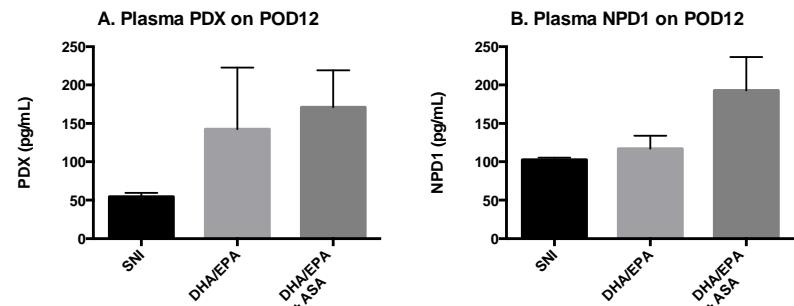


Figure 3: Perioperative supplementation with DHA, EPA, and aspirin may increase endogenous levels of neuroprotectins. A: Plasma levels of the neuroprotectin PDX trended towards increase in the DHA/EPA + ASA group compared to SNI controls on POD12 (+116.4, $p=0.07$). B: Plasma levels of neuroprotectin D1 (NPD1) trended towards an increase in the DHA/EPA + ASA group compared to SNI controls on POD12 (+90.3, $p=0.11$)

SUPPLEMENTARY MATERIAL

Statistical analysis

The areas under the “paw withdrawal threshold versus time” curve (AUC) were calculated using the trapezoid rule in Microsoft Excel and compared using a Kruskal-Wallis test with ad hoc multiplicity-corrected Dunn tests in Prism. Areas under curves have been used in clinical trials of pain as a summative measure of total pain relief and are customarily reported as percent difference from maximum AUC (28,29). For this experiment, AUC analysis is reported as mean percent of baseline paw withdrawal threshold AUC with SEM. It is thus used here as a summative measure of hindpaw sensitivity: lower AUCs correspond to increased mechanical allodynia, while those above 100% indicate lower sensitivity compared to baseline.

As another means of comparing the pain behavior of the different groups over time, centered quadratic polynomial regression analysis was performed on the behavioral data. Comparisons of best-fits were made using the Extra Sum of Squares F Test in a step-wise fashion: if comparison showed that the groups had significantly different fits, the most dissimilar group was removed from analysis until no significant difference in best fit was found between groups.

Results

Our AUC analysis showed that treatment with DHA and EPA reduced total mechanical allodynia over the course of this experiment (Figure 1B). Compared to the control SNI mice, the DHA/EPA and DHA/EPA and aspirin groups’ percent baseline paw withdrawal thresholds were increased by 47.5% and 40.3%, respectively ($p<0.05$). Again, there was no difference between the two treatment groups. This indicates that

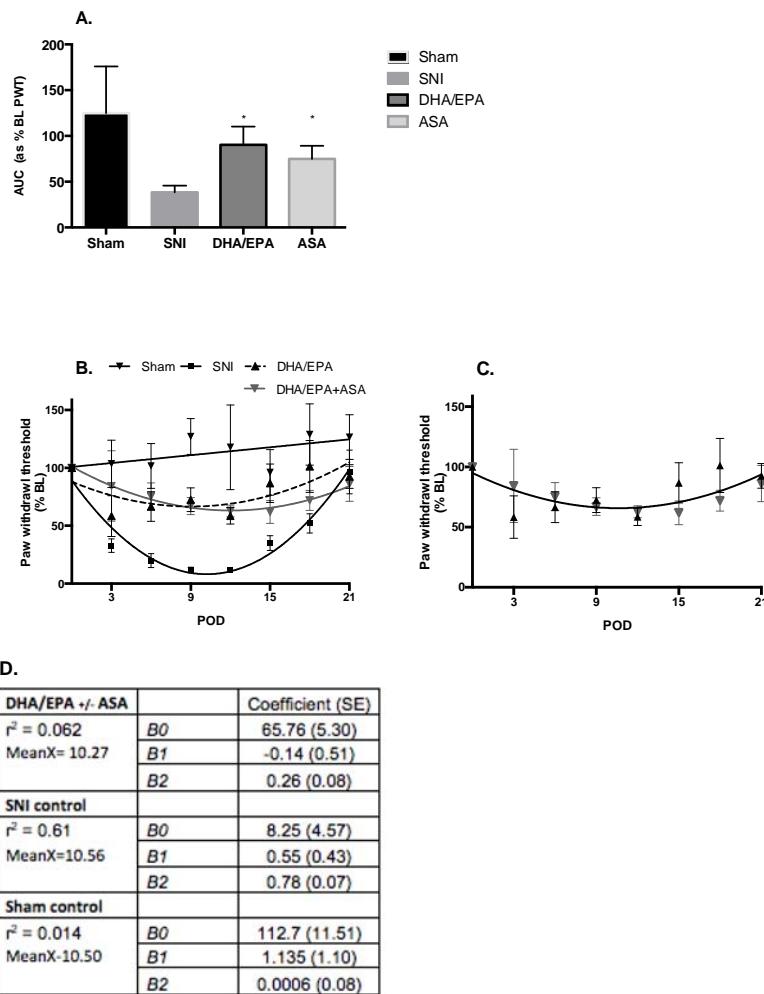
over the course of the experiment, those mice treated with DHA and EPA cumulatively experienced less mechanical allodynia after nerve injury compared to controls.

To better characterize and compare the pain behavior of the groups over time, we performed a centered quadratic polynomial regression analysis (Figures 1C-E). We found that a single model fit the data from the two treatment groups, and that this model differed significantly from the best-fit models for the sham and SNI control groups ($p<0.0001$). The fits for the treatment and sham models were poor ($r^2=0.062$ and 0.014, respectively), but this regression analysis nevertheless illustrates that the mechanical allodynia that developed in the treatment groups was less robust and progressed differently following nerve injury than in controls.

Supplementary References

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Supplemental Figures



Supplemental figure captions:

A: AUC analysis: Area under the “paw withdrawal threshold versus time” curves (AUC) for the different groups are shown as median (+IQR) percent of baseline PWT; larger values represent higher PWTs and thus less mechanical allodynia over the course of the experiment. Compared to the control SNI mice, the DHA/EPA and DHA/EPA with aspirin groups’ percent baseline paw withdrawal thresholds were increased by 47.5% and 40.3%, respectively ($p<0.05$).

B-D: Quadratic regression analysis: Comparisons of best-fits were made using the Extra Sum of Squares F Test in a step-wise fashion: if comparison showed that the groups had significantly different fits, the most dissimilar group was removed from analysis until no significant difference in best fit was found between groups. A single model fit the data from the two treatment groups, and this model differed significantly from the best-fit models for the sham and SNI control groups ($p<0.0001$). Figure C shows the initial fits for all groups; figure D shows the final fit for the two treatment groups. Figure E provides the model parameters.